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Immune Checkpoint PD-1/PD-L1: Is There Life Beyond Antibodies?

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Abstract: The PD-1/PD-L1 axis has emerged as a significant target in cancer immunotherapy. Current medications include monoclonal antibodies, which have shown impressive clinical results in the treatment of several types of tumors. The cocrystal structure of human PD-1 and PD-L1 is expected to be a valuable starting point for the design of novel inhibitors, alongside with the recent crystal structures with monoclonal antibodies, small molecules and macrocycles.

Significance The FDA-approved monoclonal antibodies targeting the PD-1 / PD-L1 axis have revolutionized the field of cancer immunotherapy. The recent increase in structural data both for small molecules and macrocycles targeting PD-1 or PD-L1 will provide valuable tools for the rational design of novel drugs against this protein-protein interaction.

1. Introduction: PD-1/PD-L1 Pathway

The development of cancer is monitored by the immune system. Most tumors are eliminated by the process of immune surveillance. In this process, T-cells play a major role; their activation stimulates an immune response against cancer cells. The T-cell activation requires two signals: a specific peptide epitope of the antigen must be presented on the major histocompatibility complex (MHC) of an antigen-presenting cell (APC) and it must form a complex with the T-cell receptor. A second signal occurring from the interaction of co-stimulatory molecules of activation is necessary. In the absence of co-stimulatory molecules, T cells enter the unresponsive state of clonal anergy (1).Tumors tend to evade immuno-surveillance by down-regulating both MHC and co-stimulatory molecules and also up-regulating co-inhibitory molecules (2). Mechanistic hallmarks by which tumors avoid immune surveillance are called immune checkpoints or co-inhibitory pathways and recently, they have emerged as a promising approach in cancer immunotherapy.

Programmed death-1 / PD-1 (or CD279) is such a member of the class of immune checkpoint receptors. It is a member of the B7-CD28 family of receptors (3). By binding on either of its two ligands PD-L1 (known also as CD274 or B7-H1) and PD-L2 (known also as CD273, B7-DC or PDCD1LG2) a co-inhibitory signal is delivered (4). PD-1 is a 55-kDa monomeric type I surface transmembrane glycoprotein. The protein is composed

of an extracellular IgV domain, a transmembrane domain and an intracellular cytoplasmic domain, which contains two tyrosine-based immunoreceptor signaling motifs; the inhibitory motif (ITIM) and the switch motif (ITSM) (5-7). Both motifs, upon PD-1 engagement can be phosphorylated and in turn recruit Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2 (8). The 40-kDa PD-L1 and the 25-kDa PD-L2 are both type I transmembrane proteins, containing extracellular IgV and IgC domains and a transmembrane domain. They lack an identifiable intracellular signaling domain (9). The two ligands share 37% identity with each other, but differ significantly in their affinity for PD-1 and their tissue specific expression.

Table 1. PD-1/PD-L1 directed FDA approved monoclonal antibodies (www.fda.gov, last update 23/9/2017)

mAb	Date of approval	Indication
Pembrolizumab (Keytruda®)	September 4, 2014	unresectable or metastatic melanoma
	October 2, 2015	metastatic non-small lung cancer
	August 5, 2016	head and neck squamous cell carcinoma (HNSCC)
	October 24, 2016	non-small cell lung cancer (NSCLC)
	March 15, 2017	refractory classical Hodgkin lymphoma
	May 18, 2017	locally advanced or metastatic urothelial carcinoma
	May 23, 2017	unresectable or metastatic, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors
	September 22, 2017	recurrent locally advanced or metastatic, gastric or gastroesophageal junction adenocarcinoma
Pembrolizumab (Keytruda®) with pemetrexed and carboplatin	May 10, 2017	previously untreated metastatic non squamous non-small cell lung cancer (NSCLC)
Nivolumab (Opdivo®)	December 22, 2014	unresectable or metastatic melanoma
	March 4, 2015	metastatic squamous non-small cell lung cancer
	October 9, 2015	metastatic non-small cell lung cancer
	November 23, 2015	renal cell carcinoma (RCC)
	November 10, 2016	squamous cell carcinoma of the head and neck (SCCHN)
	May 17, 2016	Hodgkin lymploma
	February 2, 2017	locally advanced or metastatic urothelial carcinoma
	August 1, 2017	mismatch repair deficient (dMMR) and microsatellite instability high (MSI-H) metastatic colorectal cancer
Nivolumab(Opdivo®) with **ipilimumab** (Yervoy®)	October 1, 2015	BRAF V600 wild type unresectable or metastatic melanoma
	January 23, 2016	unresectable or metastatic melanoma
Atezolizumab (Tecentriq™)	May 18, 2016	locally advanced or metastatic urothelial carcinoma
	October 18, 2016	metastatic non-small cell lung cancer (NSCLC)
	April 17, 2017	advanced bladder cancer
Avelumab(Bavencio ®)	March 23, 2017	metastatic Merkel cell carcinoma
	May 9, 2017	locally advanced or metastatic urothelial carcinoma
Durvalumab(Imfinzi ®)	May 1, 2017	locally advanced or metastatic urothelial carcinoma

2. Antibodies approved and in development

Currently, there are both antibodies targeting PD-1 and antibodies targeting PD-L1 under clinical investigation either as monotherapy or in combinations with other immune checkpoint inhibitors, monoclonal antibodies, chemotherapy, vaccines and radiation. The first monoclonal antibodies targeting PD-1 approved by FDA in 2014 were pembrolizumab and nivolumab; both for the treatment of advanced melanoma. In **table 1** an overview of mAbs in the field, approved by FDA is provided. Current focus in clinical trials aims to improve efficacy and patient response by searching for drug combinations and thus close to 1,000 clinical trials are ongoing just for checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) and its ligand PD-L1 (10).

3. Biomarkers for PD1-PD-L1

Following the clinical success of immune checkpoint inhibitors, the establishment of biomarkers in immunotherapy has emerged as an imperative need. Although dramatic survival benefits mostly for patients with melanoma and less in other types of cancers are observed, a rather small percentage of patients currently respond to PD1-PDL1 directed treatments. Therefore, biomarkers play a crucial role in predicting the likelihood of a patient's response, understanding the mechanisms of action and avoiding immune-related adverse effects (irAEs). Cancer biomarkers have been successfully established in cases of KRAS mutation, *HER2* expression and estrogen receptor expression just to name a few. Currently, PD-L1 is under investigation as a predictive biomarker of response for PD-1/PD-L1 immunotherapy.

In a recent study, PD-L1 tumor expression showed significant differences in different types of cancer. The over-expression of PD-L1 is correlated with better response to PD-1 / PD-L1 inhibition in melanoma, non-small cell lung cancer (NSCLC) and renal cell carcinoma (RCC) (11). A meta-analysis, including data from 20 clinical trials for melanoma, lung cancer and genitourinary cancers showed that in the overall sample, a significant interaction was observed between PD-L1 expression and overall response rate (ORR), which was significantly higher in PD-L1 positive patients treated with nivolumab or pembrolizumab (12). Notably, however, clinical response has also been demonstrated in patients with PD-L1 negative tumors (13).

Moreover, although the up-regulation of PD-L1 in selected solid tumors can be detected by immunohistochemistry (IHC) on both tumor and immune cells, confusion arises regarding the significance of this detection. PD-L1 is not present simultaneously on tumor and immune cells in all types of cancer (14). The fact that the expression of PD-L1 is inducible complicates the situation even further. Therefore, it is possible for PD-L1 to be expressed heterogeneously even within a patient's tumor (11). So far, the methodologies used to evaluate PD-L1 status, differ significantly. Interestingly however, in October 2015, following the accelerated approval of pembrolizumab for metastatic NSCLC, FDA approved PD-L1 IHC 22C3 pharmDx (Dako North America), as the only predictive

companion diagnostic for selecting NSCLC patients for pembrolizumab. The test's approval was based on an analysis showing that patients with at least 50% of their tumor cells expressing PD-L1 were most likely to respond to treatment. To observe the PD-L1 expression space and time resolved clearly the potential of techniques other than immunohistochemistry such as for example the modern imaging technique positron emission tomography (PET) is required.
Currently, the data concerning the potential establishment of PD-L1 as a single biomarker remain controversial. Alternative biomarker approaches, such as the quantification of tumor infiltrating lymphocytes (TILs), the identification of tumor neoantigens and the mutational load of the tumor biomarkers seem to offer a better correlation with the clinical outcomes (15).

4. Is there a need for small molecules and other approaches beyond mAbs?

There are several arguments why it is desirable to search for alternatives to mAbs in immunoncology. Generally, the production cost of mAbs remains extremely high. Moreover, the high molecular weight of mAbs, leads to poor diffusion, especially in large tumors. High affinity antibodies bind tightly to the antigen on first encounter, meaning that they remain on the periphery of the tumor, which is far from ideal for targeting solid tumors. Furthermore, the Fc portion of IgG antibodies can interact with various receptors on the surface of different cell types and thus affects their retention in the circulation (16). mAbs are immunogenic and can lead - albeit in rare cases - to irAEs sometimes with deadly outcome. The very long half-life times of PD-1 and PD-L1 directed mAbs can make irAEs difficult to treat. Small and medium sized molecules (such as macrocycles) can potentially overcome these issues. The significance of protein-protein interactions (PPIs) is well-established and although targeting PPIs with small molecules can be challenging there are successful examples of small-molecule modulators of PPIs (17).

5. Crystal structures of PD1-PDL1 and PD1-PDL2

In 2008 the first high resolution crystal structure complexes regarding this PPI were published. The complex of murine PD-1 and human PD-L1 (PDB 3BIK) (18) and that of murine PD-1 and murine PD-L2 (PDB 3BP5) (19), established the structural foundations of the PD-1/PD-L1 and PD-1/PD-L2 interactions. These structures have not allowed, however, for assessment of the extent of plasticity in these interactions when starting from the apo-protein components of the complexes. The crystal structure of the extracellular domain of human PD-1 alone was determined in 2011 (PDB 3RRQ).
Despite the fact that the murine PD-1 binds *in vitro* both to murine and human PD-L1, and human PD-1 binds to the PD-L1 of each species, it should be taken into account that the protein sequence identity between murine and human PD-1 is only 64% and between

murine and human PD-L1 is 77%. Thus, indicating likely differences in the details of the binding modes. This hypothesis was recently confirmed after Holak et al. (20) reported the crystal structure of the human PD-1/human PD-L1 complex (PDB 4ZQK, 5C3T), which indeed documents significant differences in the binding between murine and human PD-1 and the ligand (hPD-L1). This information also allowed for the identification of features of three hot-spot pockets in human PD-1/PD-L1 required for inhibition of this interaction.
PD-1 assumes a ß-sandwich immunoglobulin-variable (IgV)-type topology with Cys54 and Cys123 forming a characteristic disulfide bridge; however, PD-1 lacks the second disulfide common to other family members (CD28, CTLA-4, and ICOS).
Similarly to PD-1, the interacting, N-terminal domain of PD-L1 is also characterized by the Ig V-type topology. PD-1 and PD-L1 form a 1:1 complex within the crystal, in contrast to CTLA-4 complexes with its ligands, where both interacting partners form homodimers. The interaction of PD-1 and PD-L1 resembles that of Ig V domains within antibodies and T-cell receptors being mediated by the strands from the front faces of interacting domains (GFCC0 b sheets).
In principle, the Å-resolution crystal structure of Holak et al. (20) provides a perfect starting point for the rational structure-based drug design (SBDD) of molecules against the protein-protein interaction (PPI). However, the interface between the two proteins is rather large (~1.700 $Å^2$), hydrophobic and flat, without deep binding pockets, which makes the interface likely a difficult target for small molecules. Moreover, the hydrophobic interface also increases the chances to discover false positive hits considerably. Nonetheless, small molecule interrupters of PD1-PD-L1 have been described recently (see below).

6. Cocrystal structures with monoclonal antibodies

Recently, cocrystal structures of monoclonal antibodies targeting PD-1 or PD-L1 were described, shedding light to their molecular interactions.
For pembrolizumab, an IgG4 antibody, the crystal structure of the full-length antibody was described (PDB 5DK3) (21). The complex of pembrolizumab Fab (antigen-binding fragment) with hPD-1 (PDB 5JXE) (22) revealed that the stoichiometry is 1:1. Furthermore, the structural superposition of this complex with the hPD-1/hPD-L1 shows the overlapping surface regions, indicating that the antibody can antagonize hPD-L1 by competing for binding to hPD-1. One more crystal structure of pembrolizumab with hPD-1 (PDB 5B8C) (23) was obtained in higher resolution. It is in good agreement with the previous one and provides additional data regarding the interfacial water molecules at the binding interface, which have an impact on both affinity and specificity of the interaction. Moreover, a comparison of the crystal structure of PD-1/nivolumab Fab complex (PDB 5GGR) with PD-1/pembrolizumab (PDB 5GGS) (24) indicated that the epitopes of both antibodies occupy directly part of PD-L1 binding site and can thus outcompete PD-L1 for binding to PD-1.

Avelumab, an IgG1 antibody, utilizes in its complex with hPD-L1 (PDB 5GRJ) (25) both heavy (V_H) and light chain (V_L) to bind to the IgV domain of the PD-L1. The contribution of the light chain is greater than the heavy chain. Moreover, the binding epitope region of avelumab on hPD-L1 overlaps with the hPD-1 binding region, indicating that the partially overlapping pattern results to the blocking mechanism.

For the anti-PD-L1 mAB durvalumab, the crystal structure was recently disclosed (PDB 5XJ4). In this case both heavy and light chains contribute to the binding, resulting in steric clash which deters PD-L1 from binding to PD-1 (26).

The crystal structure of PD-L1 with BMS-936559 Fab, a fully human IgG4 antibody currently in clinical trials, showed that its epitope occupies a large part of PD-1 binding site (PDB 5GGT) (24).

In addition, in 2016 the cocrystal structure of an ultra-high-affinity engineered PD-1 mutant (HAC) with hPD-L1 was described (PDB 5IUS). This complex has a high degree of similarity with the hPD-1/hPD-L1. The main differences are observed in the β4-β5 loop. The high-affinity binding is driven by enthalpic gains, owning to the extensive polar contact network between the mutant and PD-L1 (27). In 2017 a second high-affinitiy mutant PD-1 was described, bearing a single aminoacid substitution (A132L). This leads to an increase of van der Waals interactions (28).

Furthermore, the crystal structure of a PD-L1 nanobody (single domain antibody) was published (PDB 5JDS). The nanobody KN035 competes with PD-1 for binding to PD-L1 mainly through a single surface loop of 21 amino acids (29).

In general, the binding mode seems to differ between PD-1 and PD-L1 mAbs (Figure 1). A more thorough analysis of the structural biology for PD-1/PD-L1 was recently performed by Zak et al (30).

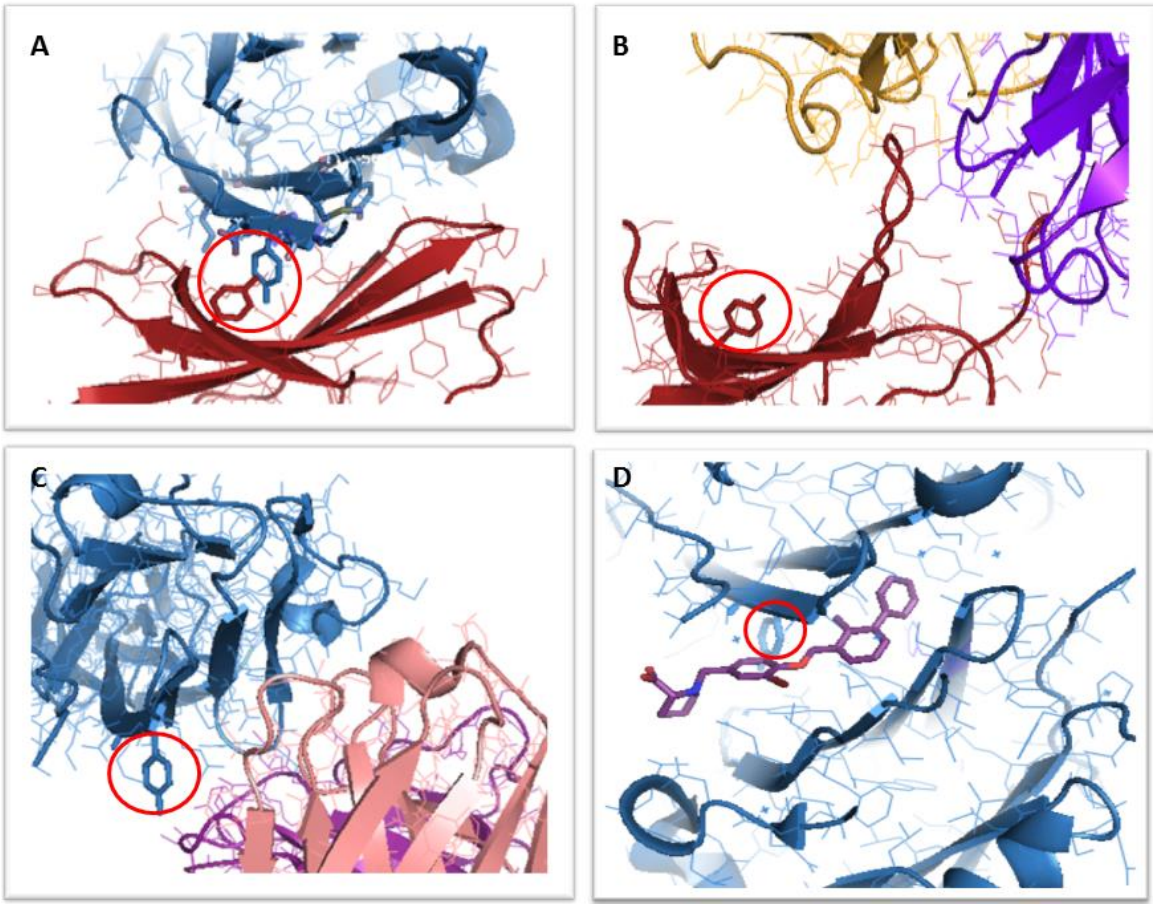


Figure 1. Different interaction modes of PD(L)1. (A) Complex of hPD-1 (red) with hPD-L1(blue) [PDB 4ZQK]. The amino acids in sticks represent the hotspots. The two residues in the red circle are Tyr68 of PD-1 (red) and Tyr123 of PD-L1 (blue). (B) Complex of PD-1 (red) with nivolumab Fab (yellow: light chain, purple: heavy chain) [PDB 5GGR]. (C) Complex of PD-L1 (blue) with avelumab Fab (purple: light chain, pink: heavy chain) [PDB 5GRJ]. (D) Complex (homodimer) of PD-L1 (blue) and BMS-08 (sticks, purple) [PDB 5J8O].

7. Cocrystal structures with small molecules

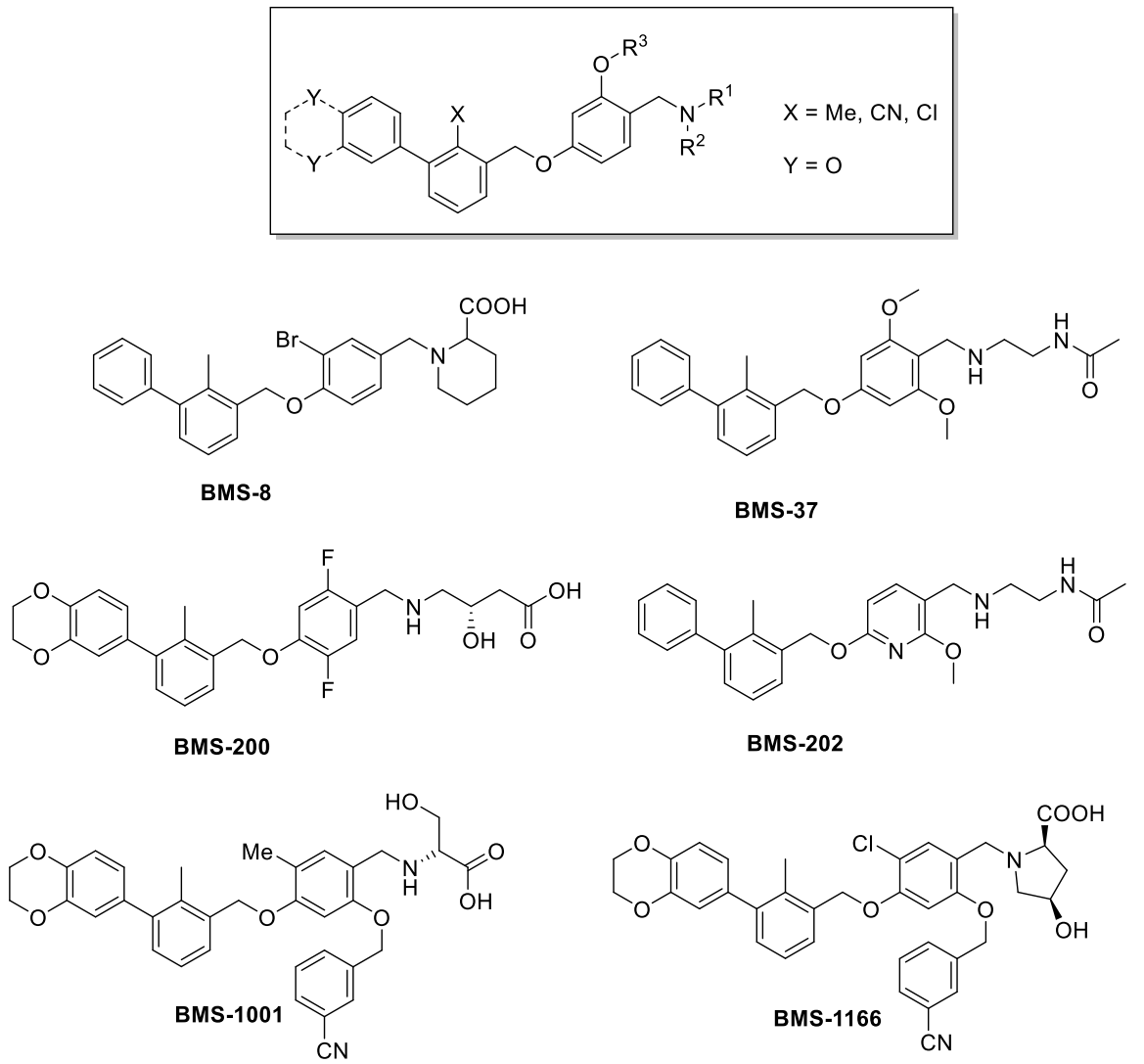
Bristol-Myers Squibb (BMS) workers have disclosed small molecules binding to PD-L1 (Scheme 1) (31). The scaffold consists of a tri-aromatic structure, including a mono-ortho substituted biphenyl substructure. Moreover, another phenyl ring is connected to the biphenyl and contains also a methylene amine moiety. The biological activity of the claimed compounds was established by a homogenous time-resolved fluorescence (HTRF) binding assay in which Europium cryptate-labeled anti-Ig was used. Typical examples are BMS-8, BMS-37, BMS-200 and BMS-202. No further *in vitro* or *in vivo* assays have been described supporting the biological activity of compounds based on the above-mentioned scaffold.

The true nature of compounds BMS-202 and BMS-8 as PD-1/PD-L1 antagonists was recently rigorously proven by co-crystal structures with PD-L1 (PDB: 5J89, 5J8O

respectively) (32). The obtained crystals diffracted at 2.2 Å resolution. Four protein molecules found in the asymmetric unit were organized into two dimers with one inhibitor molecule located at the interface of each dimer. The inhibitor inserts deep into a cylindrical, hydrophobic pocket created at the interface of two monomers within the dimer. The pocket is open to the solvent on one side of the dimer and restricted by the sidechain of $_{A}$Tyr56 on the opposite side. Overall, the inhibitor-protein interaction is best described as bimodal, spatially divided into hydrophobic and electrostatic parts following the inhibitor bimodal design.

Furthermore, Holak et al (33) disclosed two novel crystal structures of BMS-37 and BMS-200. The crystals diffracted at 2.35 and 1.7Å respectively (PDB 5N2D, 5N2F). NMR experiments indicated that both compounds bind to PD-L1 and induce its oligomerization in solution. Interestingly, the crystal structures revealed notable differences (Figure 2). The binding mode of compound BMS-37 follows the one already observed for BMS-8 and BMS-202. All of them are examples of the (2-methyl-3-biphenylyl)-methanol scaffold. However, BMS-200, an example of [3-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-methylphenyl]-methanol scaffold induced a conformational change in $_{A}$Tyr56. The 2,3-dihydro-1,4-benzodioxinyl group forces the $_{A}$Tyr56 to take a different position, thus turning the previously observed deep, hydrophobic cleft to a deep, hydrophobic tunnel and making part of the compound accessible to solvent. Two novel crystal structures were reported for the optimized derivatives BMS-1001 (PDB 5NIU) and BMS-1166 (PDB NIX) by Holak et al (34). These derivatives in particular showed significantly improved cytotoxic properties towards tested cell lines. Furthermore, it was proven that both BMS-1001 and BMS-1166 have the potential to restore the activation of effector Jurkat T cells, although less effectively than the monoclonal antibodies. Nevertheless, these data highlight the potential of small molecules.

Other small molecules have been claimed to antagonize PD1-PD-L1, however their mode-of-action has not been rigorously proven so far. An overview of claimed PD-1/PD-L1 inhibitors from patents is provided here (35).



Scheme 1. PD-1/PD-L1 inhibitors synthesized by BMS (Bristol-Myers Squibb Company).

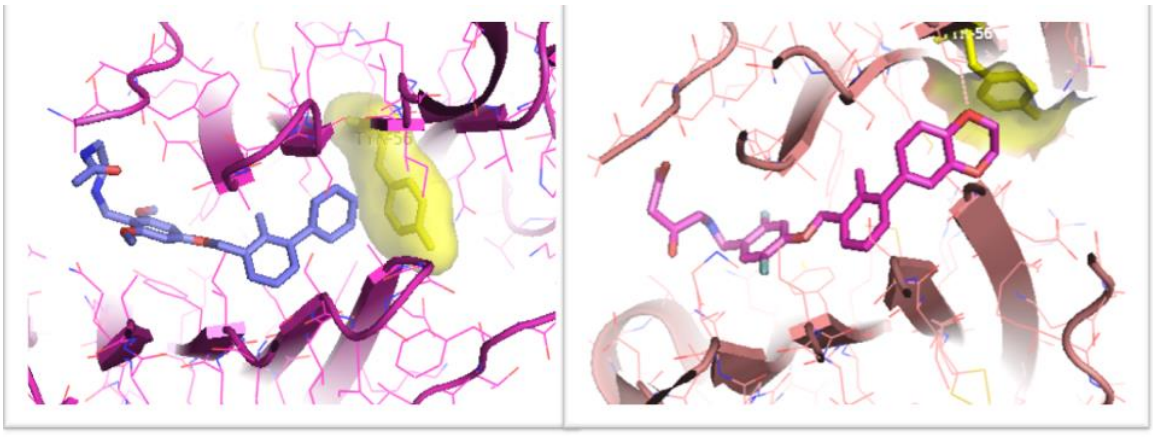


Figure 2. Binding mode of BMS-37 (left) and BMS-200 (right) on PD-L1. Yellow sticks represent $_{A}$Tyr56.

8. Macrocycles

Several patents belonging to Bristol-Myers Squibb Company claim macrocycles that showed high affinity to PD-L1 at low concentrations (36).

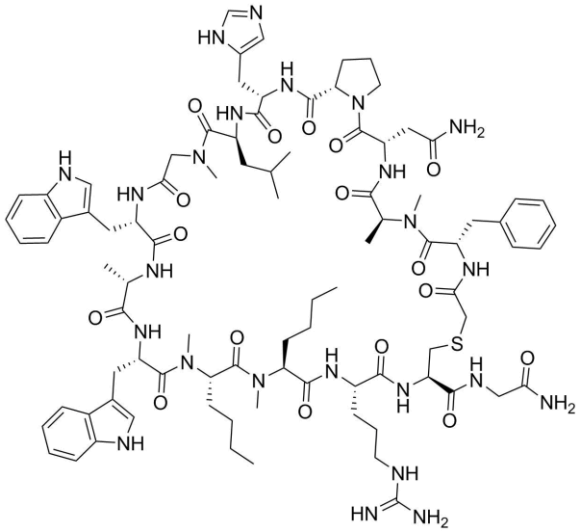
The majority of the described macrocycles contain either 14 or 13 amino acid residues [Scheme 2]. In most of them a linking sulphur atom is present and this is used as the starting point for the numbering of the amino acids. In another patent this suphur is replaced, either by oxygen or carbon.

A comparison of the different structures with the 14-motif reveals that in most cases the first amino acid is unaltered as a neutral amide or bis-amide. The alterations include the addition of extra aromatic or aliphatic rings on the amide moiety, thus making this residue more hydrophobic. The second amino acid is frequently changed and varies from a hydrophobic isoleucine to polar amino acids, including aspartic acid, arginine, lysine, serine or threonine. Amino acids 3 and 4 are mostly constant as hydrophobic moieties with chains of butane. Moreover, the backbone nitrogens in positions 3 and 4 are in almost all cases methylated. In position 5 a tryptophan is usually present or if altered it is towards a benzothiophene, a dihydropyrrole ring or an indole ring bearing a carboxylic acid substitution. Morpholine or thiomorpholine also appear, but less frequently. A highly variable position among the patents is amino acid 6, which varies from polar (serine, lysine, tyrosine, aspartic acid, glutamic acid, glutamine) to hydrophobic (alanine, glycine). Position 7 is also highly constant as a tryptophan residue, whereas position 8 is almost always a proline or a hydroxylated proline. In position 9 a usual feature is isoleucine, but it could also vary towards polar residues (aspartic acid, glutamic acid, lysine, serine, asparagines, glutamine). Amino acid 10 also varies and usually it is a polar or basic residue (histidine, lysine, morpholine, hydroxy-pyrrole, serine, asparagine, glutamine). The next two amino acids are highly constant with a proline in position 11 and an asparagine in position 12 in almost all cases. This is followed by a hydrophobic residue in position 13, usually an alanine or a proline is present. The final position 14 is always aromatic and the most common feature is tyrosine. In some cases, there are also halogens or methoxy substituents on the phenyl ring, but it seems to be less common than the tyrosine.

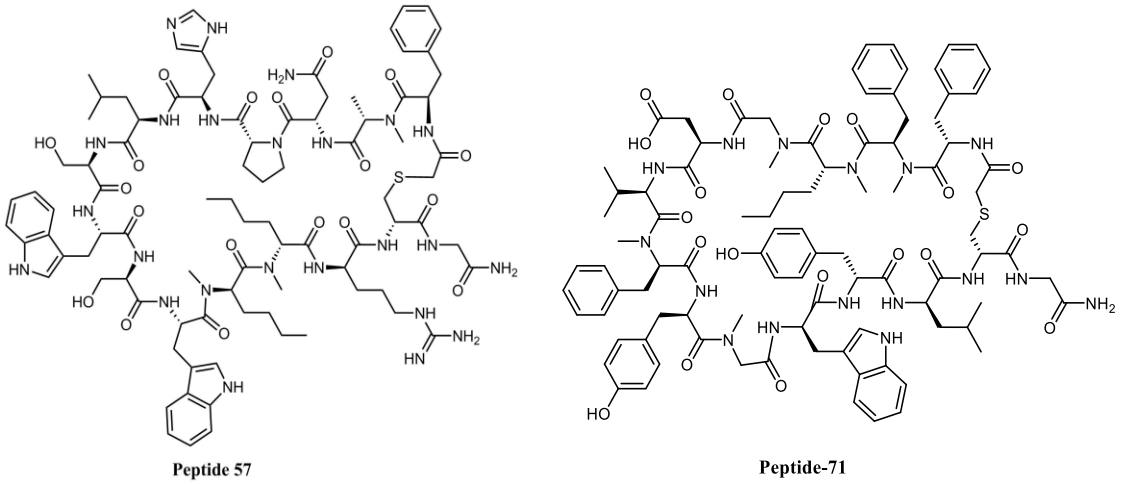
Regarding the macrocycles with 13 amino acids, the sulphur bond is always included, as well as the two proline residues in positions 5 and 10. Most likely the latter are responsible for making beta turns in the macrocycles. The main difference from the 14-motif is that there are 5 phenyl rings present (positions 3, 4, 6, 7, 12 and 13) and not three (positions 5, 7 and 14). This feature makes these macrocycles more hydrophobic. Moreover, the tyrosine which is kept constant as the 14^{th} amino acid here is always replaced with a phenyl ring with fluoro substituents.

BMS macrocyclic peptides disrupting the PD-1/PD-L1 interaction were originally studied in HTRF assay (37). Holak et al (38) studied further these macrocycles with NMR, DSF, crystallography and also in a cell assay in order to determine their ability to restore T-cell function. The analysis included peptide-57 (15-mer), peptide-71 (14-mer) and peptide-99

(13-mer). Crystal structures were obtained for peptide-57 (PDB 5O4Y) and peptide-71 (PDB 5O45) in a ratio peptide : PD-L1 1:1 (Scheme 3, figure 3). The interaction is described as “face-on binding”. In both cases there is a partially overlap with PD-1 binding epitope and the binding is dominated by hydrophobic interactions and to a smaller extent polar interactions. Closer inspection of the interactions reveals significant differences between the peptides. For peptide-57, two significant pockets are occupied with bulky indole side-chains, whereas for peptide-71 only one hydrophobic pocket is occupied with the side chain of phenylalanine. The polar interactions vary significantly between the two peptides, but in any case, the binding seems to be driven mainly by the hydrophobic interactions.. These novel crystal structures allow the comparison of the binding mode with monoclonal antibodies and provide valuable structural information for drug design.



Scheme 2. Example of macrocycle with 14 amino acids (Bristol-Myers Squibb Company WO2014151634 A1 compound 16, Ki 5nM). The numbering of amino acids starts from the position adjacent to the suphur and continues clockwise.



Scheme 3. 2D- Structures of peptide-57 (15-mer, left) and peptide-71 (14-mer, right)

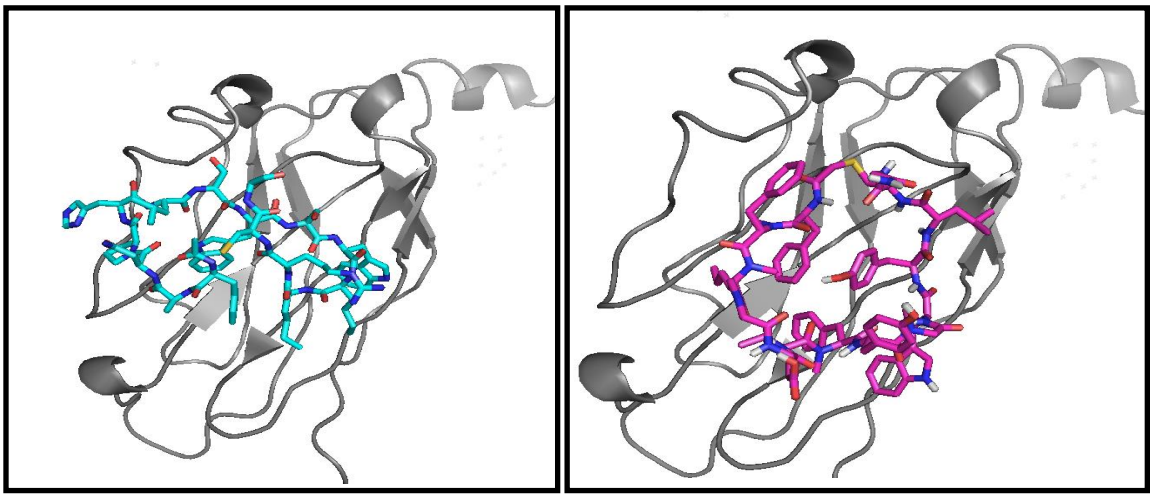


Figure 3. Binding of peptide-57 (15-mer, left) peptide-71 (14-mer, right) on PD-L1.

9. Summary and Outlook

Immune checkpoint inhibitors represent an exciting new field in cancer treatment. The disclosed crystal structures with monoclonal antibodies, small molecules and more recently macrocycles, have started to elucidate the molecular interactions.
All these data taken together provide new tools for the rational design of small molecule inhibitors, macrocycles or middle-sized cyclic peptides that may have specific advantages compared to the already approved monoclonal antibodies.

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Keywords: Programmed death-1 • PD-1 • PD-L1 • immune checkpoint • crystal structures

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